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Genetics

Positive selection of candidate tumor-suppressor genes by subtractive hybridization

(breast cancer/gap-junction protein/glutathione-*S*-transferase π/S100 protein)

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ABSTRACT **A positive selection system designed to identify and recover candidate tumor-suppressor genes is described. The system compares mRNA expression of genes from normal and tumor-derived human mammary epithelial cells grown in a special medium that supports similar growth rates of the two cell types. mRNAs uniquely expressed in normal cells are recovered as cDNAs after subtraction with mRNA from tumor cells. Seven different clones, from 0.6 to 4.8 kilobases in transcript size and including both rare and abundant transcripts, were recovered in the first 23 clones analyzed. Among the isolated clones were genes encoding the gap-junction protein connexin 26, two different keratins, and glutathione-*S*-transferase π, as well as an unknown gene in the S100 family of small calcium-binding proteins. In principle, tumor-suppressor genes include two classes: class I, in which loss of function results from mutation or deletion of DNA and class II, in which loss of function is from a regulatory block to expression. A class II suppressor gene is assumed to be regulated by a different suppressor gene that lost its function by mutation or deletion. Both classes of tumor-suppressor genes may provide valuable proteins with clinical applications in cancer diagnosis or therapy. Class II suppressors may be especially useful because the normal genes are present and their reexpression may be inducible by drugs or other treatments.**

The tumor-suppressor gene concept is >20 yr old. The concept was proposed to explain why "malignancy can be suppressed when malignant cells are fused with certain nonmalignant ones; and the hybrids resulting from such fusions produce segregants in which a loss of chromosomes is associated with reversion to malignancy" (1). The generality of these observations made in mouse cells has been demonstrated with various rodent–rodent, rodent–human, and human–human hybrids (2, 3) and with chromosome-transfer experiments (for review, see ref. 4). The results provide persuasive but indirect support for the tumor-suppressor gene concept, and challenge the investigator to identify the genes themselves, so that mechanisms of suppression can be elucidated and put to use clinically.

Despite the obvious nature of the challenge, only a handful of genes has yet been identified after years of heroic efforts by teams of talented investigators. The methods have been tedious and indirect, primarily because tumor-suppressor genes are normal wild-type genes of unknown specific function that do not lend themselves to simple assays or direct selection. The paradigm is *RB*, the retinoblastoma gene, originally identified in pediatric tumors by pedigree and cytogenetic evidence in the early 1970s (for review, see ref. 5) and later shown by transfection of the cloned gene to inhibit tumor formation in certain human *RB*-negative tumor cell lines (6, 7).

Another widely acknowledged tumor-suppressor gene is p53, initially found as a cellular protein associated with simian virus 40-encoded T antigen (8, 9). Recently, by a circuitous and indirect experimental path, p53 has been recognized as a major gene involved in many human neoplasms (10, 11).

Neither these examples nor others provide a useful paradigm for rapid selection and cloning of suppressor genes. For example, *K-REV* (12) was recovered by laborious negative selection in cell culture, Wilms tumor (*WT1*) was identified originally by pedigree analysis and cytogenetics (see ref. 4) and subsequently cloned by molecular methods (13, 14), and a gene deleted in colorectal cancer (DCC) was identified by loss-of-heterozygosity and cloned like the Wilms tumor gene by recombinant DNA procedures (15).

We describe here the use of a positive selection procedure to screen for candidate tumor-suppressor genes. The basic method, subtractive hybridization, was designed to select for genes expressed uniquely or preferentially in one of a pair of closely related cell populations (16, 17). cDNA–RNA hybridization distinguishes mRNAs that are equally expressed in both parental cell lines from those unpaired cDNAs that are uniquely expressed in the parental cells of interest.

Subtractive hybridization has particular advantages in screening for tumor-suppressor genes. Most importantly, it is a *positive* selection procedure. Irrelevant genes may also be recovered. To eliminate them as far as possible, it is important to choose very closely related cell populations as the parental pair, so that they will differ in expression primarily in genes that represent the area of interest.

Because of our interest in clinical applications, we have chosen to work with a common human neoplasm, namely breast cancer, which is the most frequent cause of cancer deaths of women in North America and Europe. Mammary carcinomas arise from epithelial cells, as do >80% of all human cancers. Of the common carcinomas, only in the mammary system can the normal epithelial cells be grown in long-term culture. Normal epithelial cells can be obtained from discarded tissue of reduction mammoplasty operations (18). Tumor samples can be obtained from biopsies and mastectomies; and putative normal mammary epithelial cells can be obtained from the same patient. Thus, the breast cancer system offers distinct advantages over other solid tumors for applying subtractive hybridization, as well as for a variety of cell and molecular studies using growing cells.

In developing this system, it was necessary to devise a medium for isolation and growth of tumor cells in which the normal cells could also be grown. The method of subtractive hybridization requires that the two parental cell populations be grown at similar rates in the same medium to minimize a background of irrelevant differences in mRNA levels resulting from disparate growth conditions. The medium we developed, DFCI-1, has the ability to support similar growth of

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both normal and tumor-derived human mammary epithelial cells (18).

Subtractive hybridization, as described (16, 17) or in modified form (19), has been applied successfully to a wide range of biological problems. Of particular relevance here are examples of selection for genes the expression of which is stimulated in (*i*) the transition from quiescence to proliferation—i.e., G_0 to G_1 (for example, ref. 20) or in the transition from G_1 to S phase (21); (*ii*) DNA damage (22); or (*iii*) stages in malignant progression (23, 24). The sensitivity of the method can be manipulated experimentally to improve one's chances of recovering rare transcripts (19, 22, 25, 26) or transcripts with a small expression difference in the two parental cell types (22). Further advantage can be gained by combination with PCR amplification (25–27).

An advantage of using cDNAs rather than genomic DNAs, as is done in screening by negative selection (12), is that cDNAs are smaller and easier to manipulate than their genomic counterpart and present in multiple copies, both in the mRNAs used for subtraction and in the cDNA libraries. Furthermore, by recovering the genes as cDNAs, the genes can be used as probes to isolate the genomic DNA, and by cloning the genes into a cDNA expression vector, a gene can be expressed directly in an appropriate cell type to produce the protein.

This paper describes the application of subtractive hybridization to the selection of tumor-suppressor genes (Fig. 1). In principle, subtractive hybridization solves the same problem of selection for a nonfunctioning gene that the penicillin method achieved for bacterial genetics (28, 29). There the problem was to select *mutants* blocked in some step of a biosynthetic pathway leading to nongrowth in a minimal medium. Here the problem is to select *genes* that are nonfunctional in tumor cells.

In our studies no special techniques were used to enrich for rare messages. Nonetheless, of the seven genes recovered in the experiment described here, one of them expressed a rare message. Many improvements in the subtractive hybridization methodology have been reported (e.g., 19, 22, 25, 26), but as yet we have not exhausted the pool of candidate genes retrieved by the methods described here.

MATERIALS AND METHODS

Cells and Culture Conditions. The normal cells used come from strain 76N, established in this laboratory from discarded reduction mammoplasty tissue from one individual and described (18). 76N cells are diploid and senesce after 15–20 passages. The tumor cells come from an aneuploid cell line (21MT-2) established in this laboratory from a pleural effusion (30, 31). In principle, the pair used for subtractive hybridization could come from a single individual, but in this instance the so-called normal mammary epithelial cells from patient 21 rapidly senesced at passage 3–4. Both parental cell populations were grown in DFCI-1 medium (18) with similar population-doubling times of ≈30 hr and harvested at 70% confluency directly into 4 M guanidium isothiocyanate/0.5 M sodium citrate/0.1 M 2-mercaptoethanol for RNA preparation (32).

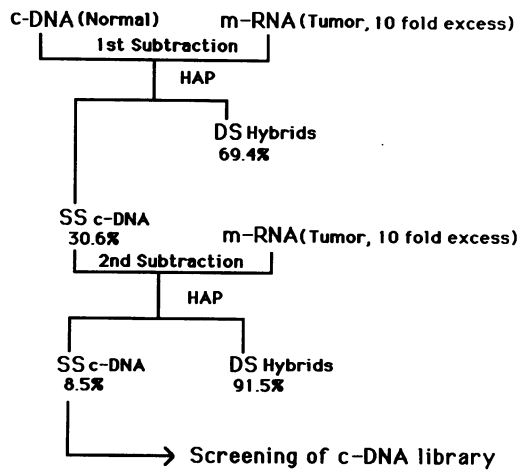

FIG. 1. Flow diagram of subtractive hybridization and yields of the recovered single-stranded cDNA. The proportion of single-stranded (SS) and double-stranded hybrid (DS) after each round of subtraction is indicated. HAP, hydroxylapatite.

Preparation of mRNA and cDNA. Total RNA was extracted from exponentially growing cells by lysis with guanidium isothiocyanate. Poly(A)$^+$ RNA was purified by two cycles of affinity chromatography on oligo(dT)-cellulose by using the standard protocol (32). The cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase (BRL) as recommended by the vendor, with the oligodeoxynucleotides $(dT)_{12-18}$ as primer (32).

Subtraction. The ^{32}P prelabeled single-stranded cDNA from 76N cells was hybridized with a 10-fold excess of tumor poly(A)$^+$ mRNA from 21MT-2 cells. Five hundred nanograms of fibronectin mRNA, prepared by *in vitro* transcription (32) was added to subtract out fibronectin cDNA, which is present at high abundance in the mRNA of the normal cells. The hybridization reaction mixture was loaded onto a hydroxylapatite column maintained at 60°C and eluted with 0.1 M phosphate buffer (pH 6.8). After rerunning the effluent through the column three times, the effluent was collected and rehybridized as above (second subtraction) without added fibronectin mRNA. The final effluent was concentrated to 200 μl, a sample was removed for quantitation, and the rest was frozen for subsequent screening.

cDNA Library Production and Screening. cDNA from 76N poly(A)$^+$ RNA was used to produce a recombinant library in the phagemid λ Zap II (Stratagene) by procedures recommended by the vendor. The 76N library was screened by differential hybridization (33) with the ^{32}P-random-primer-labeled subtracted cDNA probe against the tumor-specific cDNA. After a secondary screening the differentially expressed clones were isolated, and the inserts were amplified by PCR (34) from phage using T3 and T7 sequences as primers. After gel electrophoresis, the PCR products were purified by phenol/chloroform extraction from agarose and ^{32}P-random-primer-labeled for Northern (RNA) analysis.

Northern and Sequencing Analysis. Total RNA (20 μg) was heat denatured at 68°C for 15–20 min followed by electrophoresis in 1.2% agarose/formaldehyde gels and transferred to nylon membranes (Zeta-Probe, Bio-Rad); prehybridization and hybridization were done as described (35). Sequencing of cloned DNA was done either directly or on exonuclease III-deleted derivatives. These deletion derivatives were generated using the Promega Erase-a-Base kit. Sequencing was carried out by the dideoxynucleotide chain-termination method of Sanger *et al.* (36) with T7 DNA polymerase (Pharmacia). Parallel reactions were also performed with dGTP analogs (Pharmacia) when necessary to resolve sequence compressions.

RESULTS

Previous studies were devoted to developing the subtractive hybridization methodology for use with the mammary system (37–39). Before the 21T series of cell lines were ready for use, the parental cell populations used for subtraction were 184B5KSVTu, a nude mouse tumor-derived cell line produced in this laboratory from chemically immortalized, Kirsten sarcoma virus-infected 184 cells (40), and normal 184 cells, isolated from a reduction mammoplasty specimen (41).

Subtraction experiments, as described (38, 39) yielded three genes expressed solely in normal but not in tumor-derived mammary epithelial cells. These genes encoding fibronectin, keratin 5, and a calmodulin-related protein, gave us the first insights into the unexpected diversity of putative tumor-suppressor genes that might be found by this method. The subtractive hybridization procedure used at that time has been described (38, 39).

Subsequently we established a progression series of four tumor cell lines from the same patient (31). Two lines with primary different properties were recovered from mastectomy tissue, in which both intraductal and invasive tumor tissue was present; and two distinct metastatic lines were established later from a pleural effusion. In this report we compare mRNAs from normal cells (76N) and from one metastatic line (21MT-2) by subtractive hybridization. Metastatic cells were chosen for these experiments to provide a broad range of genetic alterations. With this particular material, however, we can in the future compare consecutive stages of tumor progression.

A library was constructed from 76N cells in λ Zap II. This excellent vector has important advantages over λ gt10 used previously. In particular, the inserts are unidirectionally ligated, production of chimeric inserts is avoided, and the inserts are easily excised at will.

In one subtraction, 50 clones were recovered, and of these 23 have been further analyzed to date. After two rounds of screening, seven different clones remained, which showed specific or highly preferential expression in the normal cells. The initial identification of these clones by Northern hybridization is shown in Fig. 2. The size range of mRNAs varied from 0.6 kilobase (kb) to almost 5 kb. Each clone has now been tested as probe against a large sample of tumor derived from nontumorigenic immortalized cell lines.

Most clones shown in Fig. 2 have now been sequenced (unpublished work). Of the others, clone 1-2 may be an estrogen-regulated gene. Clone 10 is a large (4.8 kb) mRNA expressed at low abundance. Neither clone 1-2 nor clone 10 have yet been identified. Clones 4-1 and 4-2 are different members of the keratin family. These clones include genes expressed at rare to high abundance in mRNAs.

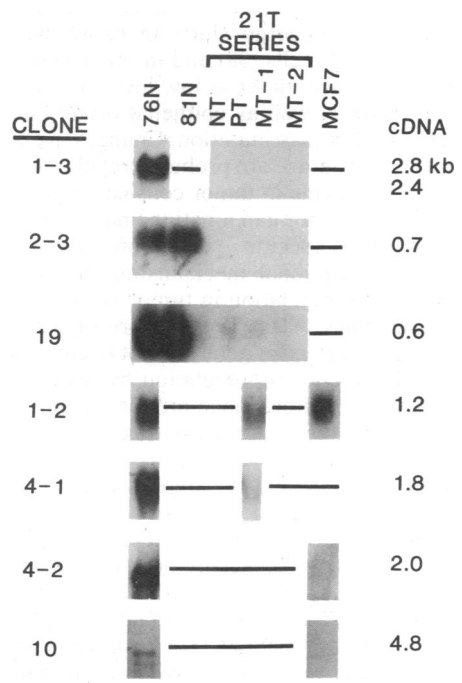

FIG. 2. Differential expression of candidate clones in RNA from normal and tumor-derived mammary epithelial cells. 76N and 81N are normal cells; the 21T series are four progressively malignant tumor lines from patient 21; MCF-7 is another mammary tumor cell line. Total RNA (20 μg per lane) was electrophoresed on 1.2% formaldehyde/agarose gels, transferred to Nylon (Zeta-Probe, Bio-Rad), and hybridized with each random-labeled probe as indicated.

Preliminary findings concerning clones 1-3, 2-3, and 19 will be summarized here and presented in detail elsewhere. Clone 1-3 is expressed in four normal strains but not in a series of tumor-derived lines (Fig. 3*A*). It has been shown by sequence comparison in GenBank to encode connexin 26, a gap-junction protein (42). Connexins are structural proteins that surround the channels of which gap junctions are composed; the channels, in turn, provide direct communication between adjacent cells (for review, see ref. 43). Gap junctions have been postulated to play a growth-regulatory role, on the basis of numerous correlations between growth control and junctional communication (for summary, see ref. 44). Of these, one of the earliest and still the most striking, is Stoker's experiment in which polyoma-transformed BHK cells were inhibited from colony formation by contact (later shown to be junctional communication) with a monolayer of normal BHK cells (45). Recent experiments by Loewenstein and coworkers (46–48) and others (49) have correlated posttranslational modulation of junctional communication with growth inhibition. Our results, in contrast, suggest transcriptional regulation, which opens the possibility for experimental and clinical modulation at the level of transcription.

Clone 2-3 encodes glutathione-*S*-transferase π, identified by sequence comparison with known genes in GenBank (50). This protein is a well-characterized enzyme present in many cell types that has detoxifying activity against many lipophilic toxic agents, including carcinogens (51). We have found that this protein is down-regulated in a number of mammary tumor-derived cell lines, both primary and metastatic, but strongly expressed in normal and immortalized mammary epithelial cells grown in culture. An example of its expression profile is shown in Fig. 3*B*.

Clone 19 represents a gene expressed in normal mammary epithelial cell strains but not in tumor-derived cell lines. Sequence comparisons have shown that it is a member of the S100 gene family, encoding small Ca^{2+}-binding proteins (≈10 kDa) with diverse functions (52). These proteins have two EF hands, domains where Ca^{2+} is bound, in contrast to calmodulin proteins, which have four. The S100 β protein is a major constituent of glial cells, whereas related proteins are expressed in differentiated but not in undifferentiated PC-12 (rat pheochromocytoma) cells (53). Clone 19 is also related in

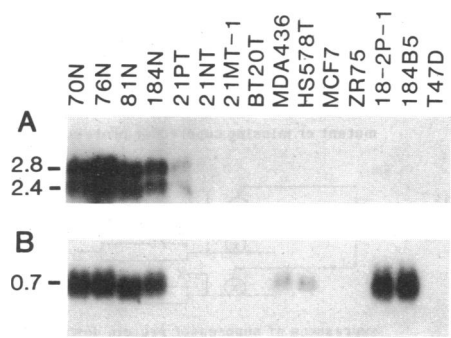

FIG. 3. RNA blot analysis of normal and tumor-derived mammary epithelial cells. 70N, 76N, 81N, and 184N are normal; all the rest are tumor-derived, except human papilloma virus-transfected 18-2P-1 and 184B5, which are immortalized cell lines. Total RNA (20 μg) from exponentially growing cells was electrophoresed on 1.2% formaldehyde/agarose gels, transferred to nylon membrane, and hybridized with ^{32}P-labeled clones. (*A*) Clone 1-3 cDNA; (*B*) clone 2-3 cDNA. The same filter was used for hybridization with both probes.

structure to another S100 family member, the small regulatory subunit of calpactin, p11 (54). MRP8 and MRP14 are S100 proteins expressed by macrophages during chronic inflammation (55). Another related protein, calcyclin, has been found in serum-induced cycling cells but not in quiescent cells, and in leukocytes from chronic myelogenous leukemia patients (56). A related mouse protein is also cell cycle induced (57). Several of these proteins have been described in rodent and human cells under different names (52). The possibility that calcyclin expression might be cancer related, as discussed in ref. 57, is particularly interesting in view of our evidence that clone 19 is not expressed in breast tumor cells.

DISCUSSION

This paper reports that subtractive hybridization experiments, in early stages, have already identified a surprisingly diverse set of candidate tumor-suppressor genes not expressed in tumor-derived cell lines compared with the normal mammary epithelial cells. Extrapolating from a small sample, our results suggest that many proteins of normal cells are absent in tumor cells.

A number of caveats should be noted. Although lack of expression suggests a suppressor function, positive evidence is necessary based on experiments in which transfected tumor lines carrying the expressing candidate gene show suppression of tumor growth, or regulated cell growth, or in which suppression is shown by other means.

Gene expression *in situ* may differ from that in culture. Nonetheless, the use of tumor cell lines grown in culture is essential for producing enough RNA for subtraction and for Northern analysis. Biopsy material used directly for RNA expression studies can be very misleading because of the heterogeneity of mammary tumor tissue that contains stromal cells as well as nonmalignant epithelial cells. After the candidate gene has been identified with cells in culture, probes and antibodies can be developed for *in situ* hybridization and immunocytochemistry using normal and tumor tissues.

Two Classes of Tumor Suppressor Genes. Classically, loss of tumor-suppressor gene expression has been viewed as the consequence of mutations or rearrangements in the gene itself. However, loss of expression of a particular gene may also result from altered regulatory events in which that gene maintains its integrity. When loss of function is assayed at the mRNA level, as in subtractive hybridization, the molecular basis may be either mutational or regulatory (Fig. 4). We refer to suppressor genes that have lost function by mutation as class I and those that are unexpressed due to a mutation occurring elsewhere as class II. The regulation of class II gene expression may be determined by a class I suppressor gene and its mutation may inhibit expression of several class II genes coordinately, as indicated in Fig. 4.

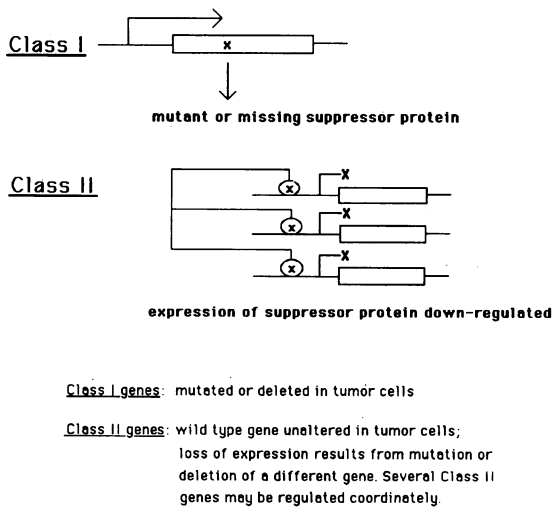

FIG. 4. Two classes of tumor-suppressor genes.

One consequence of this model is that the regulatory gene must be a positively activating regulator, which turns on expression of class II genes in normal cells. In tumor cells, however, the regulatory gene would not function, and consequently the downstream class II genes would not be expressed. In cell hybrids, the normal regulatory gene would be on, and tumor suppression would be maintained by expression of the class II downstream genes.

By these criteria, *RB*, *WT1* (Wilms tumor), and p53 are examples of class I tumor-suppressor genes. Their loss of function mutations are oncogenic (4). Some class I genes may encode trans-activating nuclear factors. Indeed, p53 (58), RB (59), and WT1 (60) proteins may be transcriptional factors and, if so, they may each control the expression of a series of class II tumor-suppressor genes. This model raises many possibilities, too extensive to discuss here. Of particular note, however, is the likelihood that each of these class I suppressors is potent because in its absence a subset of downstream class II genes are not expressed. If the entire subset is turned off coordinately, are they all required for suppression? Or can the reexpression of individual class II genes inhibit tumor formation?

Another point to note is that class II genes might, in some circumstances, undergo mutation, in which case they would become class I genes. Thus, either class I or class II genes could affect the same phenotype. One of the advantages of subtractive hybridization is that both class I and class II genes can be recovered.

The genes identified to date in our studies are probably not of class I. Although the mRNAs are absent, the genes are present in Southern blots of tumor cells. (Small deletions or rearrangements would not have been detected.) Keratins have been shown to undergo shifts in expression during immortalization of 76N cells (38) and in other systems (61). Fibronectin is expressed either at low levels or not at all in tumors of fibroblast as well as epithelial origin, suggesting down-regulation rather than mutation. Changes in expression of glutathione-*S*-transferase are probably regulatory because expression of this enzyme in tumor cells can be elevated by drug treatment (62). As for gap junction and calcium-binding proteins, their tissue-specific expression in normal cells suggests that transcriptional mechanisms may also be involved in their down-regulation in tumor cells.

Clinical Applications. Class II genes are of particular interest because the suppressor gene has not been lost and may therefore be available for up-regulation by drugs or special treatments. Despite the long-range promise of gene therapy, its use is far better adapted to circulating cells than to solid tumors. Restoration of suppressor gene function by regulatory intervention offers grand new opportunities in the design of drugs for cancer therapy.

Class II genes are immediately valuable for early diagnosis and prognosis, which are especially pressing needs in breast cancer where the course of the disease is so unpredictable. Some genes expressed differentially in normal cells may not have tumor-suppressor functions. They may, nonetheless, be very useful as diagnostic markers. When in the tumorigenic progression are these genes down-regulated? Does this occur at the same time for different genes, as if they were under coordinate control? How does the down-regulation of individual genes relate to known prognostic factors?

The candidate suppressor genes described here represent just the "tip of the iceberg" with respect to loss-of-function genes that may be significant for our understanding of the

neoplastic process, as well as useful in diagnosis, prognosis, and therapy. Genes with numerous and diverse functions may be anticipated to participate in protecting the long-lived human species from cancer. They include DNA repair genes that maintain genomic integrity and stability, genes that promote irreversible steps in differentiation, and genes that regulate proliferation. Cancer starts at the cellular level but becomes a systemic disease, and at that point, systemic mechanisms of protection play important roles. These include cell–cell communication by gap junctions, paracrine regulation by growth factors and cytokines, protection by the immune system, control of angiogenesis, and the regulation of tumor invasion. For each of these, specific genes encode key proteins the loss of which may facilitate neoplasia. It is our hope that the experimental system described here will lead us to the early recognition of key tumor-suppressor and diagnostic genes.

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